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A Simple Plate Test for Direct Visualization of Biological Lignin Degradation

(Yksinkertainen maljamenetelmä biologisen ligniininhajotuksen osoittamiseksi).

SUMMARY

A method is described, according to which lignin degradation is visualized on a complete agar medium, containing small amounts of lignin in addition to other energy sources. After appropriate incubation of the organism to be tested, the growth is scraped off and the plate is flooded with a ferrichloride-potassiumferricyanide reagent in order to visualize remaining lignin. Ability to attack lignin or lignosulfonate is indicated by colorless zones around and/or under the areas of growth. The advantages of this method as compared to the Bavendamm test for diagnostic work on lignin degradation are discussed in the light of results obtained with 52 wood-rotting and soil fungi.

TIIVISTELMÄ

Selostetun menetelmän mukaan ligniininhajotus osoitetaan täydellisellä agaralustalla, joka muiden energialähteiden lisäksi sisältää pieniä määriä ligniiniä. Sopivan kasvatusajan jälkeen kyseisen mikrobin kasvusto kaavitaan agarin pinnalta ja malja peitetään ferrikloridi-kaliumferrosyanidireagenssilla. Tämä värjää ligniinin sinivihreäksi, ja ligniininhajotus näkyy värittömänä kehänä kasvuston alla ja/tai ympärillä. Menetelmää on käytetty 52 lahottajasienen ja maaperän sienen testaamiseen, ja tulosten valossa verrataan uutta menetelmää klassilliseen Bavendammin testiin.

INTRODUCTION

For the diagnostic division of wood-rotting basidiomycetes into white-rot fungi and brown-rot fungi has for decades been used the Bavendamm test. This test makes use of the observation by Bavendamm (1), and Davidson *et al.* (2) that white-rot fungi, capable of degrading the lignin part of wood, with few exceptions, produce extracellular polyphenol oxidases detectable as deeply colored zones around the mycelium grown on agar containing certain phenolic compounds. Brown-rot fungi, that are incapable of degrading lignin, do not produce such colored zones. The same observation was made with soil-inhabiting *Basidiomycetes* (3). However, the correlation between the production of phenol oxidases and the ability to degrade lignin, does not hold for soil-inhabiting, wood-degrading *Ascomycetes* and *Fungi imperfecti* (4,5). There is also increasing evidence for the ability or inability of certain wood-degrading basidiomycetes to oxidize phenol in agar media not to be correlated with their ability or inability to decompose lignin (6,7). Fukuzumi (8) recently demonstrated that a NADH-dependent enzyme, which was isolated from a white-rot fungus, and which was probably not a phenol oxidase, was responsible for the cleavage of the aryl-alkyl ether bond of a lignin model compound.

The uncertainity concerning the role of phenol oxidases in the biodegradation of lignin has created a need for a rapid diagnostic method by which the attack on lignin as such could be evaluated, and which could be applied to wood-rotting fungi as well as to other lignin-degrading microbes.

The method to be described below has been developed to meet this requirement. It admits the testing of large numbers of organisms for lignin (or lignosulfonate) degradation without tedious chemical analyses of remaining lignin, which is inherent

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to methods up to now in use for the direct determination of lignin degradation, whether for wood blocks (9), wood foils (5), litter (10) or other natural lignin-containing material.

DESCRIPTION OF THE METHOD

Preparation of test agar. Any agar medium, suitable for the growth of the organisms to be tested may be used as base medium, if the content of phenolic and reducing compounds is low enough not to cause green coloration of the medium when plates of it are flooded with the $FeCl_3$-$K_3[Fe(CN)_6]$ reagent. Alternatively, 1 g of Difco malt extract or 1 g of Difco yeast extract *e.g.* are permissible per liter. We used the following base medium:

Glucose	5 g
NH_4-tartrate	5 g
Difco malt extract	1 g
$MgSO_4 \cdot 7\ H_2O$	0.5 g
$CaCl_2 \cdot 2\ H_2O$	0.01 g
NaCl	0.1 g
$FeCl_3$	0.01 g
Vitamin solution *)	5 ml
Agar	20 g
Distilled water	ad 1,000 ml
pH 5.8	

To 500 ml portions of molten hot medium we added 0.025—0.1 per cent (w/v) of the lignin preparation to be tested. After thorough mixing, the lignin medium was sterilized in the autoclave. Immediately after sterilization, the lignin was dispersed to a homogenous suspension with the aid of a sterile Ultra Turrax disintegrator, applied for 1 min. The homogenized test-medium was poured aseptically into a sterile Kipp dispenser flask (Fig. 1), from which 10 ml portions were poured into 9 cm Petri dishes. Alternatively, 5 ml portions can be stratified over solidified base-medium. The agar tends to foam during homogenization. Care should be taken that no foam is poured over into the dispenser flask. Homogenisation is unnecessary, if soluble lignosulfonate preparations are added.

Lignin preparations. Six preparations of lignin or lignosulfonate were used in the tests: 1) A finely ground specimen of *brown-rotted wood of Norwegian spruce*, which had been treated with boiling water and washed free of water-soluble phenolics as previously described (11). The lignin content of the sample was about 70 per cent (w/w). The sample was added in 0.1 % (w/v) concentration to the base-medium. 2) *Indulin A*, a technical lignin preparation from the West Virginia Pulp and Paper Company, Charleston A, South Carolina, U.S.A. was added in 0.05 % (w/v) concentration and 3) *"Lignin powder"*, a commercial preparation from Delta Chemicals, New York, U.S.A. in 0.025 % (w/v) concentration to the base medium, 4) *Polyfon H*, a sodium lignosulfonate preparation of the same origin as Indulin A, 5) *Peritan Na*, a sodium lignosulfonate preparation from Norcem, Oslo, Norway, and 6) a carbohydrate-free *calcium sulfite spent liquor preparation "KCL I"* (12) were likewise added to the base-medium in 0.025 per cent (w/v) concentration.

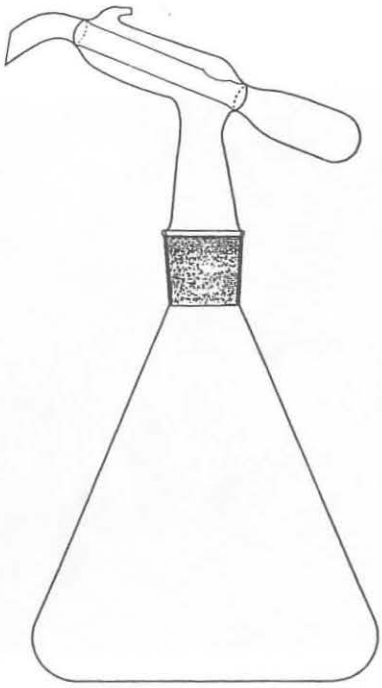

Fig. 1. Kipp dispenser flask.

Testing procedure. Test agar plates were inoculated, with not more than two fungi in each dish, and were incubated at optimal temperature, 4 to 12 days until substantial growth was noted. The points of inoculation were marked on the back of the plates. After incubation the fungal growth was scraped off with a razor blade, and the plates were flooded for 5—10 min with 10 ml of a reagent consisting of equal parts of 1 % (w/v) water solutions of $FeCl_3$ and $K_3[Fe(CN)_6]$, respectively, and mixed immediately before use (13). Strong light must be avoided. The test-agar is colored green by this reagent for phenols, and the disappearance of lignin is indicated by clear zones under and/or around the growth of lignin-decomposing fungi (Fig. 2).

*) 10 mg each of thiamine, pyridoxine, calcium pantothenate, nicotinic acid, and p-aminobenzoic acid, 2 mg of folic acid, 40 µg of vitamin B_{12}, and 20 µg of biotin in 200 ml of water.

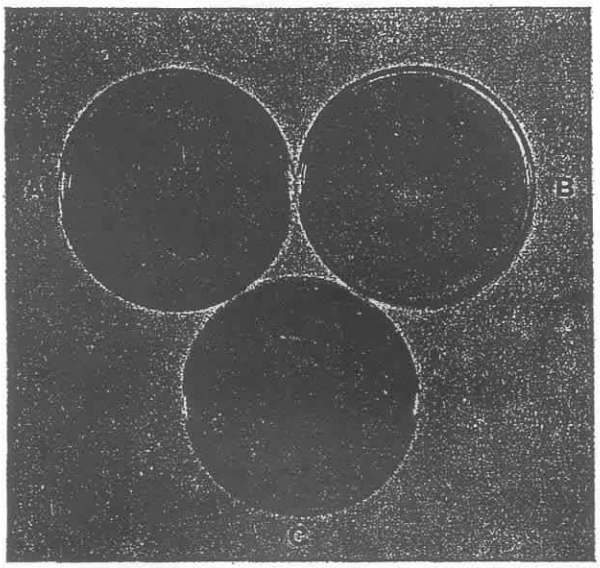

Fig. 2. Plate test for lignin degradation.

Legend: The upper plates show degradation of lignosulfonate (Peritan Na), and the lower plate of lignin (Indulin A). Each of the plates had been inoculated with two fungi as follows:

A: Upper position Fomes fomentarius
lower " Polyporus brumalis
B: upper " Chaetomium globosum
lower " Polystictus zonatus
C: upper " Lentinus lepideus
lower " P.zonatus

The inoculation sites are indicated by black dots. Remaining mycelium is seen as green coloration around inoculation sites.

RESULTS AND DISCUSSION

The method described has been applied to 52 wood-rotting or soil fungi listed in Table 1. They were simultaneously tested for the Bavendamm reaction on agar medium containing 1.5 % (w/v) malt extract and 0.5 % (w/v) gallic acid. The results are shown in Table 1. It is evident that on the whole our test for the direct visualization of lignin degradation conforms well with the Bavendamm test results.

Among the brown-rot fungi were two, *Coniophora cerebella* and *Merulius domesticus*, which according to the new test were unable to degrade any of the lignin or lignosulfonate preparations, but gave a positive Bavendamm test response. The ability of these two brown-rot fungi to produce phenol oxidases (which explain the positive results of the Bavendamm test) has been pointed out by Rösch (7).

The results of Table 1 show no contradiction between the Bavendamm test-response, and the ability to decompose at least some of the lignin preparations, among the fungi which, according to the literature, are known as white-rots. The two cultures of *Armillaria mellea* were found to be the most exacting ones as to the substrate, capable of degrading the lignin of brown rotted wood only.

Special attention was paid to the "atypical white-rots", shown by Kirk & Kelman (6) to be lignin-degrading wood-decaying fungi which are Bavendamm-negative. The results of Table 1 confirm this observation as to one of the cultures. *Polyporus dichrous* was found capable of degrading every lignin and lignosulfonate preparation tested, in spite of a negative Bavendamm test response. *Poria taxicola 105568* was demonstrated as capable of decomposing the lignosulfonate Polyfon H, whereas the other two "atypical white-rots" failed to degrade any of the lignin and lignosulfonates on the test-agar used. According to Sundman & Näse (unpublished results) lignin decomposition could be demonstrated also with these Bavendamm-negative fungi using the new plate method, if suitable growth conditions were provided.

According to Table 1 none of the wood-rotting fungi, the rot-type of which varies among different strains according to the literature, showed any attack on the substrates tested. The Bavendamm test was strongly positive with two of these fungi, *viz. Fomes roseus* and *Stereum sanguinolentum*. The malt extract, however, was darkened by the growth of these fungi even without including gallic acid into the medium.

Rot-type diagnosis based on the polyphenol oxidase activity will hence lead to erroneous results, as in the case of *Coniophora cerebella* and *Merulius domesticus* described above. According to the terminology of Kirk & Kelman (6) *F. roseus* and *S. sanguinolentum* could be named atypical brown-rots.

Among the *Ascomycetes* and the *Fungi imperfecti* of Table 1. there is an example of the non-suitability of the Bavendamm test for the evaluation of lignin degradation. *Pullularia pullulans* is unable to decompose any of the lignins tested, in spite of a strong Bavendamm reaction visible after 2 days' incubation.

The versatility of the ligninolytic ability of the white-rot fungi is obvious. More than half of these decomposed all the lignin and lignosulfonate preparations tested. The ability of *Pleurotus ostreatus* to decompose Indulin A was recently confirmed by Trojanowski & Leonowicz (18).

The method described in this paper offers a simple and feasible method for the qualitative testing of lignin degradation ability with any type of more or less modified lignin preparations, and under a variety of growth conditions, hence making it possible to include lignin degradation among characteristics covered by routine tests for identification and characterization of microbes.

Table 1. Results of Bavendamm test on gallic acid agar, and of the new direct plate test for lignin degradation.

Fungus *)	Extracellular polyphenol oxidases produced according to Bavendamm test	Lignin degradation according to the plate test with named substrates					
		Lignins			Lignosulfonates		
		Brown-rotted wood	Indulin A	Lignin powder	Polyfon H	Peritan Na	KCL 1
BASIDIOMYCETES							
Brown-rots according to literature (2, 14, 15)							
Coniophora cerebella V	+ **)	—	—	—	—	—	—
» olivacea V	—	—	—	—	—	—	—
Daedalea quercina M	—	—	—	—	—	—	—
» » V	—	—	—	—	—	—	—
Fomes pinicola V	—	—	—	—	—	—	—
Lentinus lepideus M	—	—	—	—	—	—	—
» » V	—	—	—	—	—	—	—
Lenzites saepiaria V	—	—	—	—	—	—	—
» trabea V	—	—	—	—	—	—	—
Merulius domesticus V	+	—	—	—	—	—	—
Piptoporus betulinus M	—	—	—	—	—	—	—
Polyporus betulinus V	—	—	—	—	—	—	—
» kymatodes V	—	—	—	—	—	—	—
Trametes serialis 1 V	—	—	—	—	—	—	—
» » K 2 V	—	—	—	—	—	—	—
White-rots according to literature (2, 14, 15)							
Armillaria mellea M	+	+	(+)	—	—	—	—
» » V	+	+	—	—	—	—	—
Hypholoma capnoides V	+	+	+	ng	+	+	+
Fomes annosus V	+	+	+	+	+	+	+
» fomentarius M	+	+	+	+	+	+	+
» »	+	+	+	+	+	+	+
» igniarius V	+	+	+	+	+	(+)	+
» » M	+	+	+	+	+	—	+
Lenzites betulina M	+	+	+	+	+	+	+
Marasmius androsaceus M	+	+	+	+	+	+	+
» scorodonius M	+	+	+	+	+	+	+
Panus conchatus M	+	+	+	+	+	+	+
Pholiota squarrosa M	+	+	+	+	+	+	+
Pleurotus ostreatus P	+	+	+	+	—	—	+
» » M	+	+	(+)	+	(+)	—	+
Polyporus adustus V	+	+	+	+	+	+	+
» brumalis M	+	+	+	+	+	+	+
» picipes V	+	+	+	+	+	+	+
Polystictus abietinus V	+	+	+	+	+	+	+
» versicolor V	+	+	+	+	+	+	+
» zonatus V	+	+	+	+	+	+	+
Stereum purpureum M	+	+	+	+	+	+	+
Trametes pini V	+	+	+	+	(+)	—	(+)
» versicolor P	+	+	+	+	+	+	+
Xanthochorus obliquus V	+	+	+	+	+	+	+
"Atypical white-rots" (6)							
Polyporus dichrous F	—	+	+	+	+	+	+
Poria taxicola 105568 F	—	—	—	—	+	—	—
» » 3356 F	—	—	—	—	—	—	—
Stereum frustulatum F	—	—	—	—	—	—	—
Rot type varying according to literature (2, 14, 15)							
Fomes roseus V	+	—	—	—	—	—	—
Poria vaillantii V	—	—	—	—	—	—	—
» vaporaria V	—	—	—	—	—	—	—
Stereum sanguinolentum V	+	—	—	—	—	—	—
ASCOMYCETES							
Chaetomium globosum V	+	+	+	—	—	—	+
FUNGI IMPERFECTI							
Pullularia pullulans V	+	—	—	—	ng	—	—
Stachybotrys atra V	—	—	—	—	—	—	—
Trichoderma sp. V	—	—	—	—	—	—	—

*) The origin of the fungal cultures is indicated by the letter after each name as follows:
F = U.S. Department of Agriculture, Forest Service, Forest Disease Laboratory, Laurel, Maryland, U.S.A.
M = Forest Research Institute of Finland, Department of Forest Biology, Helsinki, Finland.
P = Czechoslovac Academy of Science, Institute of Experimental Botany, Prague, Czechoslovakia.
V = The State Institute for Technical Research, Wood Preservation Laboratory, Otaniemi, Finland.

**) Code:
— = negative result
+ = positive result
(+) = questionable
ng = no growth

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